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Nitric oxide is not involved in the control of vasopressin release during acute forced swimming in rats

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Summary. Neurons of the hypothalamo-neurohypophyseal system (HNS) are known to contain high amounts of neuronal nitric oxide (NO) synthase (nNOS). NO produced by those neurons is commonly supposed to be involved as modulator in the release of the two nonapeptides vasopressin (AVP) and oxytocin into the blood stream. Previous studies showed that forced swimming fails to increase the release of AVP into the blood stream while its secretion into the hypothalamus is triggered. We investigated here whether hypothalamically acting NO contributes to the control of the AVP release into blood under forced swimming conditions. Intracerebral microdialysis and *in situ* hybridization were employed to analyze the activity of the nitrergic system within the supraoptic nucleus (SON), the hypothalamic origin of the HNS. A 10-min forced swimming session failed to significantly alter the local NO release as indicated both by nitrite and, the main by-product of NO synthesis, citrulline levels in microdialysis samples collected from the SON. Microdialysis administration of NO directly into the SON increased the concentration of AVP in plasma samples collected during simultaneous forced swimming. In an additional experiment the effect of the defined stressor exposure on the concentration of mRNA coding for nNOS within the SON was investigated by *in situ* hybridization. Forced swimming increased the expression of nNOS mRNA at two and four hours after onset of the stressor compared to untreated controls. Taken together, our results imply that NO within the SON does not contribute to the regulation of the secretory activity of HNS neurons during *acute* forced swimming. Increased nNOS mRNA in the SON after forced swimming and the increase in AVP release in the presence of exogenous NO under forced swimming points to a possible role of NO in the regulation of the HNS under *repeated* stressor exposure.

Keywords: *In situ* hybridization – Microdialysis – Hypothalamo-neurohypophyseal system – Stress

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Introduction

Nitric oxide (NO) is a freely diffusible molecule that is produced by conversion of L-arginine to citrulline via NO synthases (NOS; Bruhwyler et al., 1993; Garthwaite et al., 1989). Since many neurons have been demonstrated to contain a specific neuronal enzyme isoform (nNOS), NO has been suggested to play a pivotal role as a signal in interneuronal communication (for review see (Bruhwyler et al., 1993) and (Förstermann and Kleinert, 1995)). The study of the nNOS distribution in the rodent brain revealed that the origin of the neuroendocrine hypothalamo-pituitary system (HNS) – the magnocellular neurons located within hypothalamic supraoptic (SON) and paraventricular nuclei (PVN) – contain high levels of nNOS mRNA and protein (Calka and Block, 1993; Luckman et al., 1997). Since these magnocellular neurons synthesize the two nonapeptides vasopressin (AVP) and oxytocin (OXT), these observations raised the question as to the role NO plays in the control of the nonapeptide secretion into the blood stream. Experiments based on applying ''classical'', i.e. osmotic and reproductive, stimuli of the HNS suggested that NO inhibits the secretory activity of the HNS (Cao et al., 1996; Guevara-Guzman et al., 2000; Summy-Long et al., 1993; Thomsen et al., 1990; Villar et al., 1994).

Exposure to defined stressors modulates the expression of nNOS within neurons of the PVN (Kishimoto et al., 1996; Sanchez et al., 1999), leading to the assumption that

NO controls the activity of the major neuroendocrine stress system: the hypothalamus-pituitary-adrenal (HPA) axis. Forced swimming was found to increase the number of cells in the PVN that displayed NADPH-diaphorase (NADPH-D) activity, known to be a feature of nNOS (Sanchez et al., 1999). Recent studies reported that defined stressor exposure affects not only HPA axis activity but also the secretion of AVP and OXT from the HNS (Engelmann et al., 1999; Wotjak et al., 1998). Unfortunately, the PVN consists of both magnocellular neurons, as part of the HNS, and parvocellular neurons that constitute the HPA-axis. This makes it difficult to separate the effects on these two types of neurons, and thus, to distinguish between the two neuroendocrine systems within this nucleus. The SON, in contrast, consists exclusively of magnocellular neurons that project to the neurohypophysis and is, therefore, more suitable to study effects of defined stressor exposure on the activation of the HNS. Recent experiments revealed that under forced swimming conditions OXT, but not AVP, is released into the blood stream. This raised the question on possible inhibitory mechanisms at the level of the hypothalamus that might contribute to the unchanged plasma AVP levels (Wotjak et al., 1998).

The aim of the present study was to investigate whether NO plays a role in controlling the secretory activity of the HNS during acute forced swimming. Therefore, we used microdialysis to measure the extracellular concentration of the two products of NO production, citrulline and nitrite. Free NO in tissues is short lived and rapidly converted to nitrite. Various direct and indirect approaches have already been utilized to detect NO levels in biological samples of which a majority utilizes the more stable and therefore accumulating metabolite nitrite that results from NO produced in tissues. Such methods include the detection of nitrite by the Griess reaction (Thomsen et al., 1990) or reduction of nitrite to NO that is subsequently analyzed by chemiluminescence assays (Mayer et al., 1990). Alternatively, citrulline can be detected by HPLC (Carlberg, 1994; Chenais et al., 1991) or immunohistochemistry (Keilhoff et al., 2000; Pasqualotto et al., 1991). Here we measured both nitrite and citrulline production by microdialysis followed by chemiluminescence and HPLC, respectively, to characterize endogenous NO synthesis within the SON. Endogenous NO release was mimicked by application of authentic NO via microdialysis probes into the SON and – simultaneously – the effects on the AVP release into the extracellular fluid of the SON and into plasma were investigated. Finally, the influence of forced swimming on the nNOS mRNA levels within SON neurons was analyzed by *in situ* hybridization to monitor possible changes in the expression of nNOS within SON neurons.

Experimental procedures

Animals

Adult male rats (250–350 g b. wt.) were used in this study. All animals were kept in groups of 2–3 per rat cage under standard laboratory conditions (tap water and food *ad libitum* etc.) until surgery. All experimental procures were approved by the local Committee on Animal Health and Care.

Experiment 1: Effects of forced swimming on the release of nitrite and citrulline in the SON

Twenty two animals (Wistar strain, Tierzucht Schönewalde, Germany) were implanted with a microdialysis probe aimed at the right SON as described in detail elsewhere (Horn and Engelmann, 2001). Briefly, animals were anaesthetized with halothane and fixed in a stereotaxic frame. A microdialysis probe (U-shaped, home made) was implanted in the right SON and fixed in place with two jewelers screws and dental acrylic. After surgery, the rats were kept individually under standard laboratory conditions mentioned above. Three days after surgery the microdialysis probes of 11 animals were connected to the microdialysis assembly (Horn and Engelmann, 2001) and microdialysis probes were perfused with Ringer's solution at 3.33 μl/min. Probes were perfused for two hours to establish an equilibrium between inside and outside of the microdialysis membrane. During this period, sample collection was simulated to adapt the animals to the procedure. The collection of 30-min microdialysis samples was performed with Ringer's solution according to Fig. 1A. Forced swimming was applied for 10 min in 20°C-warm water (for details of the procedure see (Wotjak et al., 1998)) under ongoing microdialysis between 09:00 and 10:00 a.m. Immediately after swimming, the rats were returned to their home cages.

Experiment 2: Effects of direct NO administration into the SON on the release of selected amino acids and vasopressin

In addition to the microdialysis probes, a subset of 11 animals (Wistar strain, Tierzucht Schönewalde, Germany) was chronically implanted with

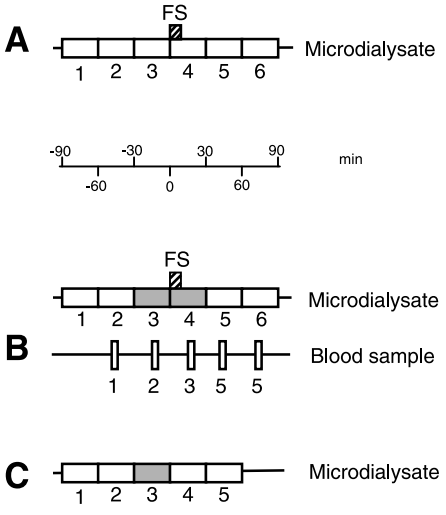

Fig. 1. Design of Experiments 1 and 2. The scheme shows **A** the number of microdialysis samples (white rectangles) collected from animals which were only implanted with microdialysis probes aimed at the SON in Experiment 2. In **B** and **C**, the sample collection of Experiment 2 is illustrated. **B** depicts the sample collection protocol for microdialysis and blood samples collected from the same animal. The microdialysis sample collection in urethane-anesthetized rats is given in **C**. FS and hatched bar = forced swimming; the numbers indicate the number of the microdialysis and blood samples; the gray boxes symbolize the duration of the NO administration via microdialysis probes

jugular venous catheters. On the experimental day (two days after surgery), catheters were connected to 1-ml plastic syringes by PE-50 tubing (Portex Ltd., UK) and microdialysis probes to the microdialysis assembly. Similar to Experiment 1, microdialysis sample collection started after the two hrs of equilibration. After this period, the collection of both microdialysis and blood samples (0.5 ml) was performed (Fig. 1B). Blood samples were withdrawn from the unrestrained rats and immediately replaced with the same volume of pre-warmed (37°C) sterile saline.

To measure the direct effect of NO on the release of AVP in the SON, six rats (Sprague-Dawley strain; Harlan, Indianapolis, U.S.A.) were implanted with microdialysis probes under urethane anaesthesia (1.4 g/kg b. wt.; Sigma, St. Louis, U.S.A.). Body temperature was maintained throughout at 37.0 ± 1.0°C, using a feedback-controlled heating blanket. Two hours after surgery and continuous perfusion of the probes with Ringer's solution, 30-min microdialysis samples were collected from the SON (Fig. 1C). NO was administered via microdialysis during one sampling interval for a duration of 30 min.

To estimate the amount of NO administered by microdialysis probes, an *in vitro* experiment was performed. Microdialysis probes (n = 5) were placed in 0.5 ml Eppendorf vials containing 100 µl of Ringer's solution. The probes were perfused with Ringer's solution containing NO for 30 min and the total amount of nitrite in both microdialysate and the Ringer's solution in the vials was measured.

Preparation of NO-solution

For application of authentic NO via microdialysis, Ringer's solution was first deoxygenated by 15-min ventilation with nitrogen gas in sealed glass flasks. The NO-stock solution was prepared by saturating this deoxygenated Ringer's solution with pure NO gas (Air liquide, Düsseldorf, Germany) by bubbling for 20 min. The final stock concentration was 1–3 mM, as measured by the Sievers 280 Nitric Oxide Analyzer (Sievers Instruments, Boulder, U.S.A.). Dilutions were made by transferring aliquots of 300 µl in gas-tight syringes from NO-stocks to 50 ml sealed flasks containing 40 ml deoxygenated Ringer's solution. This solution was then filled in nitrogen-flushed 1-ml Harvard glass syringes and mounted on the syringe pump that was used for microdialysis perfusion. The SON of the dialyzed animals were exposed to single pulses of NO-containing Ringer by switching the dialysis medium from syringes containing control Ringer's solution to Ringer's solution that contained diluted NO.

Analysis of the samples of Experiments 1 and 2

NO levels in microdialysates, in the respective blank samples, and in the Ringer's solution in the vials used in the *in vitro* study were determined using the Sievers 280 Nitric Oxide Analyzer in conjunction with the computerized data analysis program NOAWIN, as described before (Roychowdhury et al., 2001). In brief, nitrite in the biological samples was reduced to NO by potassium iodide in presence of acetic acid. For analysis of free NO in the samples, the reaction vessel was filled with water. The NO was carried from the reaction vessel to the analysis chamber by a steady flow of N_2. Chemiluminescence that resulted from the reaction of ozone with NO was measured via a photomultiplier. The instrument was calibrated by injection of different $NaNO_2$ concentrations in a fixed sample volume. Ten µl of the respective sample were injected into the purge vessel for measurement.

AVP was measured by a highly sensitive and selective radioimmunoassay (RIA; detection limit: 0.1 pg/sample; cross-reactivity less than 0.7%; (Landgraf et al., 1995; Raber et al., 1994). AVP levels in microdialysates were measured directly (Raber et al., 1994). Blank samples containing the respective dialysis medium only were treated identically to the microdialysates and showed a negligible amount of the peptide (Raber et al., 1994). Blood samples were transferred to EDTA-coated vials containing 10 µl Trasylol (Bayer, Leverkusen, Germany) and centrifuged (5000 rpm, 5 min at 4°C). The supernatant was collected and stored at −20°C until measurement of AVP content via RIA. Plasma AVP was measured after extraction (Landgraf et al., 1995).

Citrulline was determined in microdialysates by high performance liquid chromatography (HPLC) and fluorimetric detection (Waters Alliance, Milford, U.S.A.) after derivatization with o-phthaldialdehyde (OPA; Sigma-Aldrich, Deisenhofen, Germany). In the present study, 20 µl of a mixture of dialysate and OPA reaction mixture (4:1) were injected. The reproducibility of the derivatization was controlled by addition of norleucine as an internal standard. Blank samples were treated identically and showed a negligible amount of citrulline.

At the end of the Experiments 1 and 2, animals were killed by an overdose of halothane. The brains were removed, shock-frozen in dry ice-chilled n-methylbutane (Carl Roth GmbH, Karlsruhe, Germany) and stored at −80°C until sectioning with a cryocut (Leica Frigocut 2800E, Nussloch, Germany). Cresyl violet-stained coronal sections of the hypothalamus (25 µm) were used for reconstruction of the placement of the probes. Successful implantation in the SON was verified before analyzing microdialysis and plasma samples. Thus, the decision whether an animal was included into the "hit" or "outsider" groups was made before any neuroendocrine measurements were available.

Experiment 3: Effects of forced swimming on the expression of nNOS in the SON and PVN

For this experiment 15 rats (Wistar strain, Tierzucht Schönewalde, Germany) were used. Brains were obtained from control animals (n = 5) that were sacrificed without prior treatment. Ten animals were exposed to a 10-min forced swimming session identically to that mentioned above. Five animals of the forced swimming group were killed by an overdosis of halothane two hours, the other five animals four hours after onset of the stressor to obtain the brains.

After decapitation; brains were removed and immediately frozen on dry ice. Fifteen µm horizontal slices were sectioned and mounted on poly L-lysine-pre-coated slides, fixed with 4% paraformaldehyde and stored under alcohol until use. A 45-mer synthetic oligonucleotide probe (TAC ATT GGG TTG GAT CTG CTG AAC CCC AAA CGT GTT CTC TTC CAT) was used for radioactive *in situ* hybridization, corresponding to residues 349–393 of the rat nNOS mRNA (Bredt et al., 1991). The oligonucleotide probe was 3′-labeled (37°C, 5 min), using terminal deoxynucleotide transferase (EC 2.7.7.31; Boehringer Mannheim, Germany) and [α^{35}S]dATP (DuPont NEN, Dreieich, Germany, 1000–1500 Ci/mmol) according to the manufacturer's protocol, yielding a specific activity of 2×106 cpm/pmole. The brain sections were hybridized with the labeled oligonucleotide probe overnight at 42°C in hybridization buffer [50% formamide, 4 × saline-sodium citrate (SSC), 10% dextran sulfate]. Sections were then washed (1 × SSC, 30 min, 55°C), dried, and exposed to X-ray film (ßmax, Amersham, Braunschweig, Germany) for eighth weeks. As carbon-14 shows nearly the same activity as sulfur-35, co-exposed [^{14}C] micro-scales (Amersham, Braunschweig, Germany) were used to reveal a log–log linear relationship between radioactivity and optical density. Specificity of hybridization was ascertained by incubation of parallel sections with buffer containing both labeled and a 100-fold excess of unlabeled probe. These procedures resulted in images of these parallel sections indistinguishable from background. The SON was identified on X-ray films, and the optical density was measured bilaterally on two to three SON sections for each animal using SCION-IMAGE 4.02 beta (Scion Corp., Frederick, Maryland, U.S.A.).

Statistics

All data are presented as means ± SEM. For analysis of Experiments 1 and 2, the data were submitted to an one way-ANOVA for repeated measures. For the statistical analysis of Experiment 3, the average of the measurements for each animal was calculated and submitted after ln-transformation (to fit to Gaussian distribution) to an one way ANOVA. ANOVAs were followed by Fishers LSD-test if appropriate. A $p < 0.05$ was considered statistically significant.

Results

Experiment 1: Effects of forced swimming on the release of nitrite and citrulline in the SON

In 4 out of 11 animals implanted with microdialysis probes only, the dialysis membrane was found to be localized in the SON (Fig. 2A). Forced swimming failed to significantly affect the concentration of nitrite and citrulline in microdialysates in both hits and outsiders.

Experiment 2: Effects of direct NO administration into the SON on the release of vasopressin and citrulline

The data of the *in vitro* study revealed that 5.5 ± 0.2 nM of NO were administered during a 30-min dialysis period.

In all 11 animals, the microdialysis membranes were localized in the SON. Administration of NO via microdialysis strongly increased intra-SON citrulline (to approx. 500% of baseline, $p < 0.01$) after swimming and returning to the regular Ringer's dialysis medium (Fig. 3A). For plasma AVP measurements, samples of one animal had to be discarded because of two missing values. In the remaining five animals plasma AVP was increased both during NO administration alone and in combination with forced swimming (to approx. 150%, $p = 0.03$; Fig. 3B). Without NO treatment, intra-SON citrulline and plasma AVP remained unchanged throughout the experiment ($p > 0.05$; Fig. 3A, B).

ANOVA revealed a significant treatment effect of NO on intra-SON AVP release as measured in microdialysates (to approx. 300%, $p < 0.05$; Fig. 4).

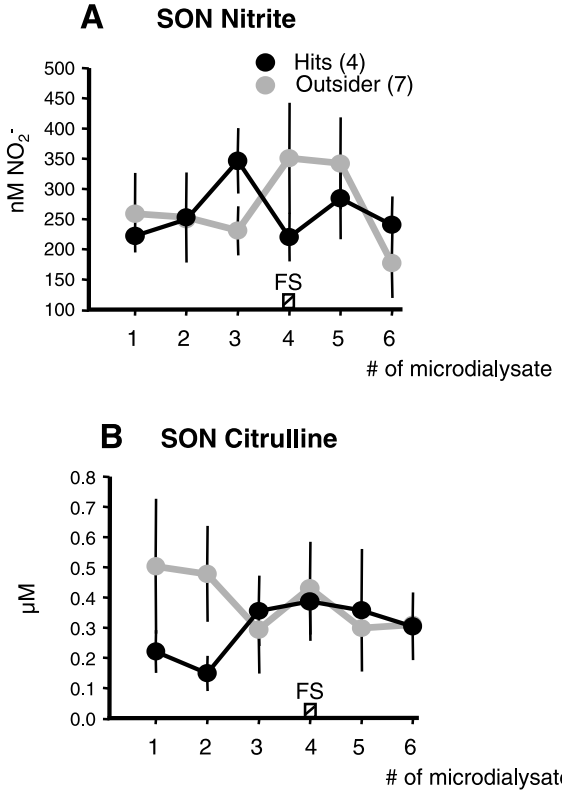

Fig. 2. Concentration of (**A**) nitrite and (**B**) citrulline (means ± SEM) measured in the same microdialysates from the SON before, during (FS and hatched bar), and after forced swimming. Numbers of microdialysates correspond with those given in Fig. 1A

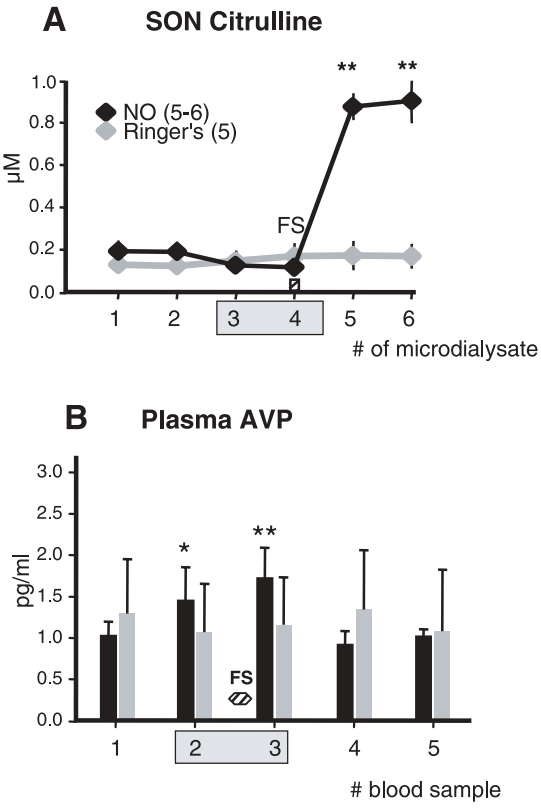

Fig. 3. Concentration of (**A**) citrulline in microdialysates (means ± SEM) obtained from the SON before, during, and after forced swimming (FS and hatched bar) of animals that received either Ringer's solution or Ringer's solution containing NO (gray box) via microdialysis probes. Concentration of AVP in plasma samples (**B**; means + SEM) obtained from the same animals. Numbers of microdialysates and plasma samples correspond with those given in Fig. 1B. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus previous samples of the same treatment group

Experiment 3: Effects of forced swimming on the expression of nNOS in the SON and PVN

Statistical analysis revealed a significant effect of forced swimming on the density of silver grains corresponding

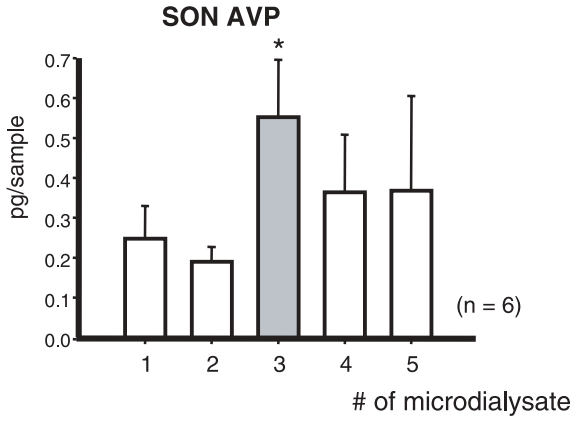

Fig. 4. Concentration of AVP in microdialysates (means + SEM) obtained from animals which received Ringer's solution containing NO via the microdialysis probes in the right SON. Numbers of the blood samples correspond with those given in Fig. 1C. $^{*}p < 0.05$ versus samples 1 and 2

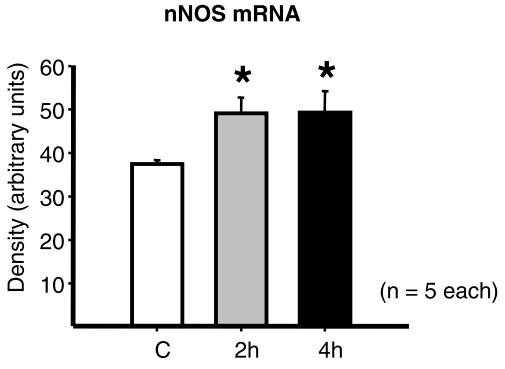

Fig. 5. Hybridization signal (expressed as arbitrary units) of nNOS mRNA measured on X-ray films within the hypothalamic supraoptic nucleus (means + SEM). Rats were killed two (*2 h*) and four (*4 h*) hrs after onset of a 10-min forced swimming session. Control animals (*C*) were killed under control conditions without stressor exposure. $^{*}p < 0.05$ versus C

with nNOS mRNA in the SON two and four hours after the onset of the stressor ($p < 0.05$; Fig. 5).

Discussion

Our data show that forced swimming does not increase the concentration of nitrite or citrulline, a major by-product by the conversion of arginine to NO (Hemmens and Mayer, 1998), in the extracellular fluid of the SON. This indicates that NO is not released from magnocellular cells of the SON under forced swimming conditions. The resulting hypothesis that NO is not involved into the inhibition of the peripheral AVP release during acute forced swimming was tested by investigating the secretory response of vasopressinergic neurons in the SON to the exposure to exogenous NO. This approach revealed that NO administration triggered the release of AVP into plasma under control conditions and in combination with forced swimming (see Fig. 3B). Furthermore, NO administration triggered the release of AVP within the SON (see Fig. 4). These findings imply that, at the level of the SON, exogenous NO stimulates rather than inhibits the release of AVP from both axon terminals and somata/dendrites of vasopressinergic HNS neurons.

Interestingly, NO administration combined with forced swimming triggered also the release of citrulline within the SON (see Fig. 3A). Given the suggested coupling between NO synthesis and the production of this amino acid (Hemmens and Mayer, 1998) and their correlated release (Guevara-Guzman et al., 2000), this finding suggests that NO administration stimulates NO synthesis. Based on previous studies, one can envisage a cascade in which NO causes the release of excitatory amino acids (Garthwaite et al., 1989) and other putative neurotransmitters which in turn may cause elevated intracellular Ca^{2+}-levels. Increases in intracellular Ca^{2+} comprises a trigger for NO synthesis via nNOS and is Ca^{2+}-dependent. Therefore, our finding of increased citrulline levels supports the hypothesis of self-sustaining stimulation of NO production. The stimulatory action of the NO treatment in the SON suggests that under *acute* forced swimming conditions, NO is not causally involved in the regulation of the secretory activity of vasopressinergic HNS neurons.

It could be argued that our approach to measure the release of endogenous NO by microdialysis failed simply because of methodological limitations. However, two arguments do not support this suggestion. First: the detection limit of both our NO-detector and the HPLC for citrulline are significantly lower than the concentrations measured in the microdialysates (detection limit nitrite: 50 nM, lowest measured: 150 nM; detection limit citrulline: 0.01 μM, lowest measured: 0.1 μM). Second: the data of Experiment 2 demonstrate that significant changes in extracellular citrulline caused by NO-administration in combination with forced swimming can be easily detected with our methods (Fig. 3).

It has been shown that restraining and/or immobilization increases nNOS mRNA levels mainly in the parvocellular part of the PVN (Kishimoto et al., 1996; Krukoff and Khalili, 1997). Furthermore, forced swimming increased the immunoreactivity for NADPH-D in the PVN (Sanchez et al., 1999). In contrast to the PVN, little is known about the action of NO in the SON. The results of Experiment 3

demonstrate that forced swimming increases the expression of nNOS within the SON in adult male rats. Although it has been reported that restraining increases nNOS mRNA within the SON (Krukoff and Khalili, 1997), it is difficult to define the role that NO may play after its release from magnocellular neurons within the SON. Possible functions range from modification of the local blood supply via actions on presynaptic endings on input neurons to the magnocellular neurons and actions on glial cells to autocontrol mechanisms of the secretory activity of the SON neurons themselves. Two recent studies suggest that NO inhibits the excitability of vasopressinergic and oxytocinergic neurons, partially due to a potentiation of the GABAergic input (Engelmann et al., 2002; Stern and Ludwig, 2001). Further studies are needed to provide a deeper insight into the local NO actions also with respect to the postulated modulation of the behavioral consequences in response to forced swimming exposure by NO (Jefferys and Funder, 1996).

Different lines of evidence imply an involvement of intrahypothalamic NO in HNS regulation in response to ''classical'' HNS activation induced by osmotic and reproductive stimuli for (see Kadekaro and Summy-Long, 2000 for review and Srisawat et al., 2000). In contrast, the data presented here suggest that within the SON, NO is not involved in the physiological response to the fast and *acute* change in HNS regulation induced by forced swimming. Since mRNA encoding nNOS was increased in the SON after forced swimming, further studies are needed to verify a possible role of an increased NO production in the regulation of the HNS under *repeated* stressor exposure.

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